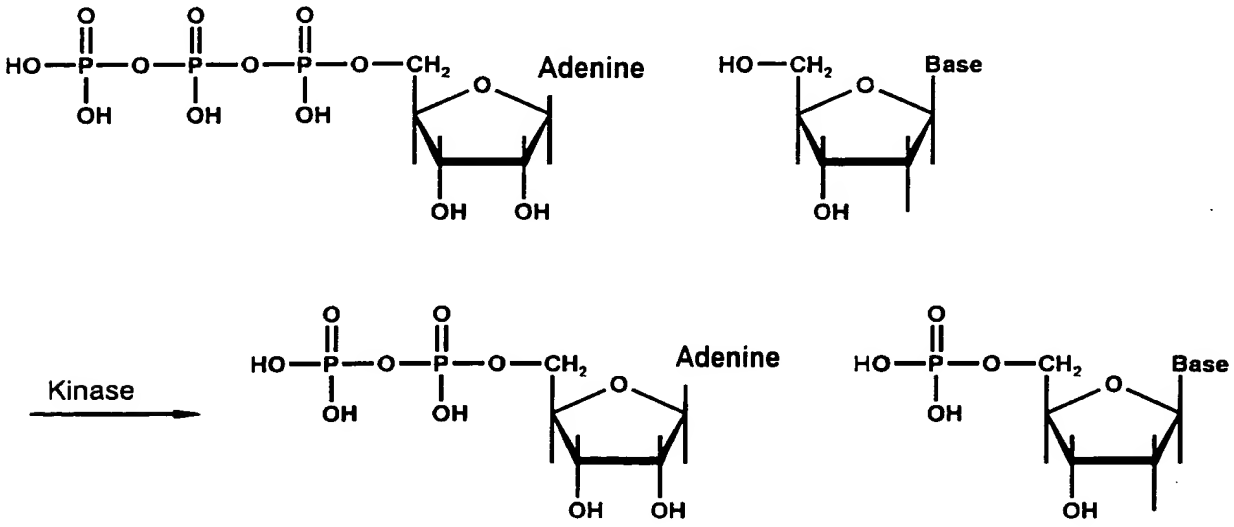
Roche Diagnostics GmbH **4862/OA/**

Deoxynucleoside kinase from insect cells for the synthesis of nucleoside monophosphates

The subject of the present invention is a recombinant kinase from insect cells such as e.g. Drosophila Melanogaster, remaining stable during the synthesis of nucleoside monophosphates without the addition of stabilizing SH reagents, without stabilizing proteins and detergents and accepting all four natural deoxynucleosides. A further subject matter of the present invention is a DNA sequence encoding the kinase according to the invention as well as a procedure for preparation of the kinase according to the invention and its use during the synthesis of nucleoside monophosphates.

(Deoxy)-nucleoside kinases catalyze the phosphorylation of nucleosides or deoxynucleosides, respectively, to the corresponding nucleotide monophosphates and have therefore an important role in the "salvage pathway" of the nucleotide metabolism.

The catalyzed reaction is:



The deoxynucleoside monophosphates are starting products for the deoxynucleoside triphosphates which are used to a very increasing extent as reagents for the PCR reaction.

The deoxynucleoside monophosphates are at present accessible by three ways:

1. from hydrolysis of fish sperm
2. by chemical synthesis from deoxynucleosides
3. by enzymatic synthesis from deoxynucleosides.

The hitherto known methods have a number of disadvantages. Thus, during the hydrolysis of fish sperm all 4 monophosphates are produced in about the same quantities; this is a fact that misses the requirements of the market (e.g. d-UTP, partially used instead of d-TTP is prepared from d-CTP). In addition, d-TTP, resulting from hydrolysis, is contaminated with approx. 2% d-UTP and can, practically, not be isolated.

Furthermore, the animal origin of the educts has to be assessed as critical from a regulatory point of view (GMP). Moreover, the market of monophosphates from fish sperm is very limited.

A number of side products are produced during the chemical synthesis which are difficult to separate by chromatographic purification. In addition, several bases (e.g. guanosine) must be provided with protective groups before phosphorylation which increases the synthesis time considerably.

The disadvantages of the state of the art were overcome by the provision of a recombinant multifunctional deoxynucleoside kinase from insect cells such as, in particular, Drosophila melanogaster (Dm-dNK) remaining stable during the synthesis of nucleoside monophosphates without the addition of stabilizing SH reagents, without stabilizing proteins and detergents and accepting all four natural deoxynucleosides: thymidine (dThd), deoxycytidine (dCyd), deoxyadenosine (dAdo) and deoxyguanosine (dGuo). In the present invention stable means that the yield rate for the catalyzed reaction does practically not decrease within 5 hours, preferably 10 hours, particularly preferably within 12 hours at 37°C. It is surprising that the enzyme remains

stable for such a long time without addition of stabilizers containing thiol. This stability has not been observed in other kinases until now (1-9). By leaving out these stabilizers when using the kinase according to the invention in the synthesis the synthesis gets cheaper and, above all, the product purification can be simplified to a great extent.

Furthermore, hitherto known kinases have a considerably higher substrate specificity; as a consequence, for the synthesis of the individual nucleosides it is no more necessary to have the corresponding specific kinase. Particularly advantageous is the low specificity for the synthesis of modified nucleoside analogues, such as dideoxynucleosides or base- or sugar-modified nucleosides. Base-modified nucleosides are for example 7-deaza-nucleosides, C-nucleosides and nucleotides labelled with reporter groups (dye, digoxigenin, biotin) at the base. Sugar-modified nucleosides are for example azathymidine, arabinosyl-thymidine. The kinetic constants of the Drosophila kinase compared to known analogous enzymes are listed in table 1. The specific activity kc of the kinase according to the invention is several times higher than that of the kinases known before. The activity of the enzyme was measured as described in the reference: Munch-Peterson et al. (1991) J.Biol.Chem. 266, 9032-9038. By this, a considerably lower amount of enzyme is necessary to synthesize the dNMPs. (factor 3.5 – 14000, cf. Kc values in table 1). The specificity constant (k_c/K_M) of the kinase according to the invention exceeds that of the hitherto known kinases by several powers and is in the region of the diffusion constant. This leads to the complete yield when the kinase is added to the d-NMP synthesis. They are higher by factor 2-6500 than the hitherto known kinases, s. fig. 1.

Surprisingly, the enzyme according to the invention is still stable at 60°C what is advantageous for the reaction procedure. Preferably, the enzyme according to the invention has at T=37°C a half-life of $t_{1/2} \geq 50$ h in Tris buffer with 5 mM $MgCl_2$ and $t_{1/2} \geq 25$ h in water and accepts all natural deoxynucleosides (example 6).

A further subject matter of the invention are kinases from other non-vertebrate organisms, in particular from other animal species of the Hexapoda class showing comparable properties to those of the Drosophila kinase. Particularly such kinases essentially having the above described stability and the above described substrate specificity. Peferred kinases are those isolated from

the subclass of Pterygota and particularly preferable are those from the Diptera class, particularly preferable from the Drosophilidae family.

A further subject matter of the invention is a DNA sequence as well as functional fragments thereof coding for the kinase according to the invention. The DNA sequence according to the invention is characterized in that the primers listed in the following hybridize onto the DNA sequence of the kinase according to the invention:

SEQ ID No.: 2

GGGAAGTGGCAGGAGTAGCTCCCG

SEQ ID No.: 3

CTCCCGTTGTAG**CCG**TCGCCCTTCTGG

SEQ ID No.: 4

GACGACTGGCTCGGG**CAG**CTCTTCACCGCG**TTG**

SEQ ID No.: 5

TTCGATTTTTATTACCTCGCGAGGTAA

SEQ ID No.: 6

AGGTAA**AAA**TCGCGAGCGATA**ACG**AAGCAC

SEQ ID No.: 7

CACCGCATGCTTGCGTAGGCCGTCGCCCGAGCAAGACTCCTC

SEQ ID No.: 8

GACTACATGTTTCTAGGGTTCTTCACC

A further subject matter of the invention are also such kinases and DNA sequences onto the DNA sequence of which hybridize oligonucleotides with the SEQ ID No.: 2, 3, 5, 7 and 8 or with the SEQ ID No.: 2, 4, 5, 7 and 8 or with the SEQ ID No.: 2, 5, 6, 7 and 8.

The following hybridization conditions are advantageous:

- Hybridization: 0.75 M NaCl, 0.15 Tris, 10 mM EDTA, 0.1% sodium pyrophosphate, 0.1% SLS, 0.03% BSA, 0.03% Ficoll 400, 0.03% PVP and 100 µd/ml boiled calf thymus DNA at 50°C for approx. 12 hours.
- Washing: 3x30 minutes with 0.1 X SET, 0.1% SDS, 0.1% sodium pyrophosphate and 0.1 M phosphate buffer at 45°C

The kinase sequence according to the invention is given in fig. 5, SEQ ID No.: 1.

The DNA sequence according to the invention is obtainable from Drosophila Melanogaster by the procedure described in the following:

A pBluescript SK +/- phagmide containing a 1.1 kbp cDNA insert which contains among others the presumed gene coding for the deoxynucleoside kinase was obtained from the Berkeley Drosophila genome sequencing project (clone LD15983). The first 600 base pairs of the 5'end of the 1.1 kbp cDNA cloned via EcoRI and XhoI in the multiple cloning site (MCS) of the phagmide were already sequenced by Harvey et al., University of California, Berkeley. Based on these sequence information new primers were designed (Dm-TK1 and Dm-TK2/SEQ ID NO.**9**: 5'TCCCAATCTCACGTGCAGATC-3' and SEQ ID NO **10**: 5'-TTCATCGAAGAGTCCATTCAC-3' which enabled complete sequencing of the insert. Dm-TK1 is a 21 bp sense primer binding upstream from the presumed translation start region. Dm-TK2 was designed as 21 bp sense primer according to the 3' region of the cDNA part already sequenced.

With this sequence an open reading frame including 750 bp and coding for a protein with 250 amino acids could be identified. The DNA sequence SEQ ID NO.**1** is depicted in figure 5. The calculated molecular weight of this protein was 29 kDa and corresponds therefore to the data given by Munch-Peterson et al. 1998 indicating a weight of nearly 30 kDa for native Dm-dNK.

Starting from the sequence information the structure gene coding for the Dm-dNK could be isolated from the 1.1 kbp cDNA insert of the pBluescript SK +/- phagmide by the "polymerase

chain reaction" technique (PCR) (Mullis, K.B. and Faloona, F.A., Methods in Enzymol. 155 (1987) 335-350). For this, a specific primer pair (see SEQ ID NO. **11**: 5'-GCGCGAATTCATGGCGGAGGCAGCATCCTGTGC-3' and SEQ ID NO.**12**: 5'-GCGCAAGCTTATTATCTGGCGACCCTCTGGC-3') with the corresponding endonuclease restriction site of the later insertion into appropriate expression plasmids was synthesized. Thus, the 5'-primer (Dm-dNK3) has an EcoRI endonuclease restriction site upstream from the coding sequence whereas the 3'-primer (Dm-dNK4) contains a HindIII endonuclease restriction site downstream from the coding sequence. Furthermore, the 3'-primer contains downstream from to the coding sequence two stopcodons for safe termination of the translation. Further, suitable primers which were optimized for the translation initiation in E. coli were the following:

SEQ ID No.: 13 (Dm-dNK 3)

5'-CGCGAATTCA TGGCGGAAGC GGCGAGCTGC GCGCGTAAGG GGACC-3'

SEQ ID No.: 14 (Dm-dNK 4)
5'-CGCAAGCTTA TTAACGGGCG ACCCTCTGGC-3'

Cloning of the structure gene for the Dm-dNK in pUC18

The PCR preparation was applied to an agarose gel and the 750 Bp structure gene was isolated from the agarose gel. The PCR fragment was cut with the EcoRI and HindIII restriction endonucleases for 1 hour at 37°C. Simultaneously, the pUC18 plasmid was cut with the EcoRI and HindIII restriction endonucleases for 1 hour at 37°C, the preparation was then separated by agarose gel electrophoresis and the 2635 Bp vector fragment isolated. Subsequently, the PCR fragment and the vector fragment were ligated by T4-DNA-ligase. Then 1 µl (20 ng) of vector fragment and 3µl (100 ng) of PCR fragment, 1µl 10x ligase buffer (Maniatis et al., 1989 Molecular cloning, a laboratory manual, Sambrok, Fritsch, Maniatis, Book 3, Section B27; Munch-Peterson (1991) J. Biol. Chem. 266, 9032), 1 µl T4-DNA-ligase, 4 µl sterile H_2O bidist . were pipetted, carefully mixed and incubated over night at 16°C.

The cloned gene was checked by means of restriction analysis and by sequencing.

Cloning of the structure gene for the Dm-dNK in appropriate expression vectors

For expression of the Dm-dNK the structure gene was cloned in appropriate expression vectors in such a way that the structure gene is inserted in the right orientation under the control of an appropriate promoter, preferably an inducible promoter, particularly preferably the lac-, lacUV5-, tac- or T5 promoter. Preferred expression vectors are pUC plasmids with lac- or lacUV5 promoters or pKK plasmids.

For this, the structure gene was cut out of the plasmid pUC18 for the Dm-dNK by means of EcoRI and HindIII, the restriction preparation was separated by agarose gel electrophoresis and the approx. 750 Bp fragment was isolated from the agarose gel. Simultaneously, the expression vectors were cut with EcoRI and HindIII, the restriction preparation was separated by agarose gel electrophoresis and the resulting vector fragment was isolated from the agarose gel. The resulting fragments were ligated as described. The appropriate insertion of the gene was verified by restriction analysis and sequencing.

Preferred expression vectors are also pUC18, pKK177-3, pKKT5. Especially preferred is pKKT5. The expression vector pKKT5 is obtained from pKK177-3 (Kopetzki et al. 1989, Mol. Gen. Genet. 216:149-155) by exchanging the tac- promotors with the T5-promoter derived from the plasmid pDS (Bujard et al. 1987, Methods Enzymol. 155:416-433). The EcoRI-endonuclease restriction site was removed from the sequence of the T5-promotor by point mutation.

Transformation of the expression vectors in different E-coli expression strains

Competent cells of different E. coli strains were prepared according to the Hanahan method (J. Mol. Biol. **166** (1983) pp. 557). 200 µl of the resulting cells were mixed with 20 ng of isolated plasmid DNA (expression vectors). After 30 min. incubation on ice a thermal shock (90 sec. at 42°C) was carried out. Subsequently, the cells were transferred in 1 ml LB-medium and incubated for phenotypical expression for 1 hour at 37°C. Aliquots of this transformation pre-

paration were plated on LB plates with ampicillin as a selection marker and then incubated for 15 hours at 37°C.

Appropriate host cells are E. coli K12 JM83, JM101, JM105, NM522, UT5600, TG1, RR1ΔM15, E.coli HB101, E.coli B.

Expression of Dm-dNK in E.coli

For the expression of Dm-dNK clones containing plasmid were inoculated in 3 ml Lb_{amp} medium and incubated in the shaker at 37°C. At an optical density of 0.5 at 550 nm the cells were induced with 1mM IPTG and incubated in the shaker for 4 hours at 37°C. Subsequently, the optical density of the individual expression clones was determined, an aliquot with an OD_{550} of 3/ml was taken and the cells were centrifuged (10 min. at 6000 rpm, 4°C). The cells were resuspended in 400 µl TE buffer (50 mM TRIS/50 mM EDTA, pH 8.0), released by ultrasound and then the soluble protein fraction was separated from the insoluble protein fraction by centrifugation (10 min., 14000 rpm, 4°C). A buffer containing SDS and β-mercaptoethanol was added to all fractions and the proteins were denatured by boiling (5 min. at 100°C). Subsequently, each quantity of 10 µl was analyzed by means of a 15% analytical SDS gel (Laemmli U.K. (1970) *Nature* **227**: pp. 555-557).

A further subject matter of the invention is a method for production of the nucleoside monophosphates which is characterized in more detail by the following steps:

- Synthesis of the nucleoside monophosphates starting from nucleosides by enzymatic phosphorylation with a kinase according to the invention as an enzyme
- Use of a nucleotide triphosphate as a phosphate group donor in catalytic amounts
- In situ regeneration of the phosphate group donor via a regenerating system (CK/CP; PK/PEP; acetylphosphate/acylkinase, pyrophosphate/pyrophosphorylase)

As a nucleoside monophosphate according to the invention the original nucleoside monophosphates, deoxynucleoside monophosphates, dideoxynucleoside monophosphates as well as other sugar- and base-modified nucleoside monophosphates are applicable.

A further subject matter of the present invention is the use of the kinase according to the invention in the synthesis of the nucleoside monophosphate.

Brief description of the figures:

Figure 1:

The kinetic constants of different nucleoside kinases are listed in figure 1 (hTK1/2 = human thymidine kinase ½; hdCK=human deoxy-cytidine kinase; hdGK=human deoxy-guanosine kinase; HSV=Herpes Simplex Virus). The data are taken from:

a) Munch Petersen et al. J. Biol. Chem. 266, 9032 (1991); J. Biol. Chem. 268, 15621 (1993),
b) Biochem. Biophys. Acta 1250, 158 (1995),
c) Bohmann and Eriksson Biochemistry, 27 4258 (1988),
d) Wang et al. J. Biol. Chemistry 268, 22847 (1993),
e) Iwatsuki et al. J. Mol. Biol. 29, 155 (1967),
f) Black et al. J. Gen. Virology 77, 1521 (1996),
g) Ma et al. P.N.A.S. 93, 14385 (1996).

Figure 2:

Figure 2 shows the formation of d-CMP from cytidine under the conditions mentioned in example 2.

Figure 3:

Figure 3 shows the formation of d-AMP from adenosine and d-GMP from guanosine under the conditions mentioned in example 4.

Figure 4:

Figure 4 shows the formation of d-CMP from cytidine under the conditions mentioned in example 3.

Figure 5:

Figure 5 shows the DNA sequence of the clone.

Figure 6:

Figure 6 shows the temperature optimum of the nucleoside kinase from D. Melanogaster.

Figure 7:

Figure 7 shows the stability of the recombinant Dm-nucleoside kinase compared to isolated Dm-nucleoside kinase. Figure 7A was determined without addition of BSA, fig. 7B with addition of BSA.

References

1. Lee, L.-S. and Y.-C. Cheng (1976) Human deoxythymidine kinase. I. Purification and general properties of the cytoplasmic and mitocondrial isozymes derived from blast cells of acute myelocytic leukemia, J. Biol. Chem., 251, 2600-2604.

2. Cheng, Y.-C. and M. Ostrander (1976) Deoxythymidine kinase induced in HeLa TK cells by herpes simplex virus type I and type II. II. Purification and characterization, J. Biol. Chem., 251, 2605-2610.

3. Ellims, P.H., T.E. Gan and L. Cosgrove (1982) Human thymidine kinase: Purification and some properties of the TK1 isoenzyme from placenta, Mol. Cell. Biochem., 45, 113-116.

4. Gan, T.E., J.L. Brumley and M.B. Van Der Weyden (1983) Human thymidine kinase. Purification and properties of the cytosolic enzyme of placenta, J. Biol. Chem., 258, 7000-7004.

5. Sherley, J.L. and T.J. Kelly (1988) Human cytosolic thymidine kinase. Purification and physical characterization of the enzyme from HeLa cells, J. Biol. Chem., 263, 375-382.

6. Munch-Petersen, B., L. Cloos, G. Tyrsted and S. Eriksson (1991) Diverging substrate specificity of pure human thymidine kinases 1 and 2 against antiviral dideoxynucleosides, J. Biol. Chem., 266, 9032-9038.

7. Bohman, C. and S. Eriksson (1988) Deoxycytidine kinase from human leukemic spleen: Preparation and characterization of the homogenous enzyme. Biochemistry, 27, 4258-40265.

8. Kierdaszuk, B. and S. Eriksson (1990) Selective inactivation of the deoxyadenosine phosphorylating activity of pure human deoxycytidine kinase: Stabilization of different forms of the enzyme by substrates and biological detergents, Biochemistry, 29, 4109-4144.

9. Kristensen, T. Quantification of thymidine kinase (TK1) mRNA in normal and leukemic cells and investigation of structure-function relationship of recombinant TK1 enzyme. 1996.

Department of Life Sciences and Chemistry, Roskilde University, Denmark. Ref Type: Thesis/Dissertation. Available at Roskilde University Library.

10. Hanahan D., (1983) *J. Mol. Biol.* **166:** 557

11. Sambrook J., Fritsch E.F., Maniatis T., (1989) *Molecular cloning: A Laboratory Manual second Edition*, B.27 Cold Spring Harbor Laboratory Press NY (USA)

12. Mullis, K.B. und Faloona, F.A., (1987) *Methods in Enzymol.* **155:** 335-350

13. Munch-Peterson B., Piskur J. und Sondergaard L. (1998) J. *Biol. Chem.* **273,** 3926-3931

14. Laemmli U.K. (1970) *Nature* **227**: pp. 555-557

The invention is further explained by the following examples:

Example 1:

Production and isolation of the recombinant Dm kinase

An E. coli strain BL21 was transformed with a pGEX-2T vector (Amersham Pharmacia Biotec), in which the structure gene of the Dm kinase was cloned, by means of the $CaCl_2$ method (Sambrook, Molecular cloning, 2nd ed. Cold Spring Harbor Laboratory press). A transformed colony was suspended in 100 ml LB medium (10 mg tryptone, 5 mg yeast extract, 8 mg NaCl per l), containing 50 µg/ml ampicilline, over night at 37°C. The next day, the culture was adjusted to an OD of 0.6 in 1 l of LB medium and the expression was induced by 100 µl IPTG. The culture temperature of 25°C was maintained over night and the cells were gathered by centrifugation. The cells were resuspended in 100 ml of buffer A (20 mM of potassium phosphate (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 10% glycerin, 1% Triton X100 and 0.1 mM phenylsulfonylfluorides). The mixture was broken up by the French press. The homogenized substance was centrifuged (20000 rpm/15 min.) and filtered with a 1µm Whatman glass micro filter and a 0.45 µm cellulose acetate filter.

The homogenized substance was applied to a GSH column (15 x 45 mm), equilibrated with 10 column volumes of buffer B (140 mM NaCl, 2.7 mM KCL, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 1 mM DTT, 10% glycerol, 1% Triton X100, 0.1, mM phenylsulfonylfluoride, 5 mM benzamidine, 50 mM aminocaproic acid). The column was washed with 50 bed volumes of buffer B and 10 volumes of buffer C (140 mM NaCl, 2.7 mM KCl, 10 mM NaH_2PO_4, 1.8 mM KH_2PO_4) and afterwards the fusion protein was split by recirculation of 1 column volume of buffer C with 400 U thrombin for 2 hours. The Dm-nucleoside kinase was then eluted with 3 column volumes of buffer C.

Example 2

Comparison of synthesis of d-CMP with and without thiol addition

d-Cyt	22 mg
Tris buffer pH 8.0	2 ml
MgAc	10 mg
ATP	66 mg
d-NK	0.132 U
DTT	7 mg/0 mg

The yield is determined by the integration of the peak areas using HPLC.
The reaction is not considerably slower in the preparation without DTT and is, above all, not terminated after 45 hours (see figure 2).

Example 3

Synthesis of d-GMP and d-AMP

d-Ado or d-Guo	28 mg
Water	2 ml
MgAc	32 mg
ATP	3 mg
CK	100 U
d-NK	0.396 U
CP (creatinphosphate)	20 mg

The reaction advances for 32 hours without slowing down, the yield rate is above 80% and no thiols must be added. The addition of Tris buffer is not absolutely necessary (see figure 3).

Example 4

Synthesis of d-CMP

d-Cyt	22 mg
Tris buffer pH 8.0	2 ml
MgAc	32 mg
ATP	3 mg
CK	100 U
d-NK	0.132 U
CP	20 mg

Even after 66 hours the enzyme is still active despite lacking thiol stabilizers. The yield rate is 80% despite the use of only catalytic ATP amounts (see figure 4).

Example 5

Synthesis of NMPs, dd-NMPs and base-modified d-NTPs

Substrate solution

CP	250 mg
ATP	7 mg
Mg acetate	160 mg

in 25 ml 50 mM Tris pH 8.0

An amount of each 0.5 ml of the solution is added to approx. 2.5 mg of the corresponding nucleoside. Then 50 U of the creatine kinase and 0.32 U of d-NK are added.

Preparation	Nucleoside	Time	Yield rate
a)	Cytidine	15 h	90%
b)	dd- adenosine	70 h	40%
c)	Iso-guanosine	2 h	80%

Example 6

Activity of the kinase from D. Melanogaster at different temperatures

The activity of the Dm-nucleoside kinase was determined at different temperatures. It shows a wide optimum with a maximum at 60°C (see figure 6).
The activity test is described in reference No. 14.

Example 7

Activity of the recombinant Dm-kinase compared to native Dm-kinase.

The activity of the recombinant Dm-kinase compared to native, isolated Dm-kinase was determined. After different periods of incubation in 50 mM Tris pH 7.5 + 2.5 mM $MgCl_2$ at 37°C the remaining activity was determined.

Whereas the recombinant Dm-kinase remains stable without the addition of BSA the activity of the native kinase decreases within 50 min to < 20%. By adding 2.5 mg/ml BSA the native kinase remains stable as well (fig. 7A + 7B).

The half-life in Tris buffer in the presence of $MgCl_2$ is 50 h, without $MgCl_2$ 31 h and in pure water 28 h. The native Dm-kinase has a half-life of <12 min. under the same conditions.

INFORMATION FOR SEQ ID NO. 9 (DM-dNK1)

SEQUENCE CHARACTERISTICS:

(A) LENGTH : 21 base pairs

(B) TYPE : nucleic acid

(C) STRANDNESS : single

(D) TOPOLOGY : linear

5′-TCCCAATCTCACGTGCAGATC-3′

INFORMATION FOR SEQ ID NO. 10: (DM-dNK2)

SEQUENCE CHARACTERISTICS:

(A) LENGTH : 21 base pairs

(B) TYPE : nucleic acid

(C) STRANDNESS : single

(D) TOPOLOGY : linear

5′-TTCATCGAAGAGTCCATTCAC-3′

INFORMATION FOR SEQ ID NO. 11: (DM-dNK3)

SEQUENCE CHARACTERISTICS:

(A) LENGTH : 33 base pairs

(B) TYPE : nucleic acid

(C) STRANDNESS : single

(D) TOPOLOGY : linear

5′-GCGCGAATTCATGGCGGAGGCAGCATCCTGTGC-3′

INFORMATION FOR SEQ ID NO. 12: (DM-dNK4)

SEQUENCE CHARACTERISTICS:

(A) LENGTH : 33 base pairs

(B) TYPE : nucleic acid

(C) STRANDNESS : single

(D) TOPOLOGY : linear

5′-GCGCAAGCTTATTATCTGGCGACCCTCTGGC-3′